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<b>13. ABSTRACT (Maximum 200 Words)</b>  The final goal of our project is to elucidate the apoptotic and cellular senescence mechanisms of tumor suppression in breast cancer. p53 is clearly involved in both apoptosis and senescence, which has led to our emphasis on the p53 pathway. Although the initial approach was a genetical study of the p53-dependent apoptosis pathway, we shifted the focus of study to cellular senescence program, based on our findings of the senescence associated heterochromatic foci (SAHFs). We further characterized SAHFs and found that SAHF-formation is dependent on the p16/Rb pathway using stable retroviral RNAi system. We also found that E2F-targets, which are required for cell cycle progression and regulated by the Rb-family, are stably silenced in senescence. We propose that E2F-targets are heterochromatinized possibly by being involved in SAHFs, resulting in the 'irreversible' cell cycle arrest in senescence.				
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## Introduction

The objectives outlined in our original proposal were designed to elucidate the mechanism of the tumor suppression through the p53-dependent apoptosis pathway, using a genetic approach. As already mentioned in the first annual report and the New Statement of Work, we shifted the focus of the research from apoptosis to senescence. Senescence appears to involve both the Rb and p53 tumor suppressor pathways (1-3). Hence, as cells enter a senescent state, p53 and p16 accumulate, and Rb becomes hypophosphorylated. In breast cancer, approximately 50 % of tumors show low or no p16 expression, and many have mutations in Rb or p53. It is also significant that cells derived from mammary tissues almost always lose p16 expression when cultured *in vitro* (4). These observations may indicate a higher propensity of mammary cells, compared to other cell types, to inactivate the senescence pathway by losing p16. The p16/Rb pathway is also important for the formation of heterochromatin during senescence, which we recently designated Senescence Associated Heterochromatic Foci (SAHFs) (5; attached paper). The recent discovery that histone H3 Lys9 methylation (H3-Lys9-methyl) is the hallmark of heterochromatin conferred the molecular basis for the heterochromatic structure (6). Of the modifications of histones, methylation is extremely stable and apparently irreversible. Based on these findings, we hypothesized that the epigenetic determination of senescence-specific gene expression patterns may be important for maintaining the stable growth arrest in senescence.

## **Body of Annual Report**

Senescence was initially identified as a permanent form of cell cycle arrest that accompanied the replicative exhaustion of human fibroblasts in culture. However, recent work from our laboratory and elsewhere has shown that senescence is induced by many forms of cellular stress. By analogy to apoptosis, we have been seeking the "common machinery" of cellular senescence. A large body of evidence indicates that both the p53 and Rb tumor suppressor pathways contribute to cellular senescence. Our laboratory and others reported that p53 is required for the induction of 'premature' senescence by oncogenic ras (1, 2). However, p53 is not essential for maintaining senescence (7). On the other hand, there are several lines of observation, which suggests the involvement of epigenetic regulation of gene expression upon senescence (8). Furthermore, we just reported that senescent human fibroblasts exhibit the condensed chromatin structure, which shares heterochromatic features, and that the formation of these structures was dependent on the presence of an intact p16/Rb pathway (attached paper and First annual report).

### **Characterization of chromatin structure in senescent HDFs (attached paper; Figs. 1-3)**

As we described in the first annual report, senescent HDFs exhibit dramatic heterochromatic structures. We changed the term for this structure from SAHRs to SAHFs (senescence associated heterochromatic foci) for the publication (5; attached paper).

### **E2F-targets are heterochromatinized in senescence (attached paper; Figs. 4, 5)**

The alteration of chromatin organization during senescence could be related to senescence-specific changes of gene expression. We focused on E2F-targets, because these genes are essential to the cell cycle progression, and inactivation of the Rb family, which are co-repressors of E2F-targets, by E1A completely abrogates the senescence process. Chromatin immunoprecipitation analysis revealed the accumulation of HP1 $\gamma$  and histone H3-Lys9M, hallmarks of heterochromatin, on the promoter regions of some E2F-targets (cyclin A and PCNA). Furthermore, we also showed that adenoviral transduction of E2F-1 transcription factor in senescent cells showed lower expression of E2F-targets than quiescent cells, indicating that these promoters are less accessible to transcription factors during senescence. These data show that E2F-targets are stably silenced in senescence, which could contribute to the irreversibility of cell cycle arrest in senescence.

### **Mechanism of SAHF-formation (attached paper; Figs. 6, 7)**

The adenovirus E1A oncoprotein can interfere with the induction of SAHFs in a manner that depends on its ability to inactivate Rb, implying that an intact Rb pathway is required for the process. To further dissect the mechanism of SAHF-formation, we designed short hairpin RNAs (shRNAs) against p16 and Rb using retroviral gene transfer in collaboration with Dr. Greg Hannon (CSHL). This allows us to stably knockdown the genes of interest. To specifically knockdown p16, we designed the target sequence in the exon 1 $\alpha$ , because exon 2-3 are shared with another gene, p14arf. We tested multiple shRNAs for each gene and selected the one, which showed the highest reduction of the protein level by Western blotting. Knocking down p16 and Rb levels could not prevent the cell cycle arrest by oncogenic ras. However, the SAHF-formation was blocked, at least partially, by these shRNAs. At the same time, some E2F-targets were up-regulated by these shRNAs, indicating the upregulation of these genes is not the natural consequence of cell proliferation. Furthermore, although cells co-expressing sh-p16 or sh-Rb with oncogenic ras were heterogeneous, individual cells, which failed to form SAHFs, expressed high levels of MCM3, one of the E2F-targets, while cells with SAHFs exhibited very low levels of MCM3. Hence, a close correlation exists between SAHF-formation and the repression of E2F-target genes through p16/Rb.

#### **Generation of HP1 knockout mice**

The putative role of HP1 proteins in maintaining senescence is consistent with a tumor suppressor activity and, consistent with this view, HP1 $\alpha$  is not expressed in a subset of breast cancers (9). In collaboration with Dr. Harald Von Melchner (University of Frankfurt), we have obtained ES cells that have insertional mutations in the HP1 $\alpha$ . So far, three lines of HP1 $\alpha$ +/- ES cells have produced chimeric mice. Unfortunately, however, none of them have not had germ line transmission. In the mean time, different HP1 $\alpha$ +/- ES cells have been injected. After getting the HP1 $\alpha$  KO mice, we will characterize the chromatin structural changes in different tissues, gene expression pattern alterations, and *in vivo* tumorigenesis (breast cancer model).

#### **Generation and characterization of the new anti-H3 K9M antibodies**

Histone H3-Lys9 can be mono-, di-, or tri-methylated *in vitro* and *in vivo*. However, most available antibodies against H3-Lys9 methyl cannot discriminate these modifications. Furthermore, these anti-H3Lys9 methyl antibodies cross react with H3 Lys27 methyl, which is also involved in the gene silencing (10).

##### **i) Generation of rabbit polyclonal anti-Lys9 tri-methyl Ab**

KLH-conjugated polypeptide corresponding to amino acids 6-13 (TAR[tri-methyl-K]STGG-C)

was used as an antigen. Anti-serum recognized both H3 Lys9 di- and tri-methyl. To obtain the H3 Lys9 tri-methyl specific Ab, anti-serum was first passed through di-methyl peptide column. Flowthrough of di-methyl peptide column then was applied to tri-methyl column, followed by elution with glycine (pH 2.5). Specificity to H3 Lys9 tri-methyl was confirmed by ELISA using non-modified H3 Lys9 peptide, H3 Lys9 di-, and tri-methyl peptides (Fig. 1).

ii) Generation of mouse monoclonal anti- Lys9 di-methyl Ab

We also tried to generate monoclonal Lys9-dimethyl Ab using the same polypeptide with di-methylated Lys9. One test serum specifically recognized the Lys9 di-methyl (Fig. 1). We have not successfully obtained the stable hybridoma cell lines.

iii) H3 Lys9 di- and tri-methyl in heterochromatin

H3 Lys9 tri-methyl specific Ab immuno-staining showed punctuated pattern in MEFs. These H3 Lys9 tri-methyl enriched foci are clearly overlapped with DAPI-densely stained foci, which are pericentric heterochromatin (Fig. 2A). On the other hand, H3 Lys9 di-methyl Ab showed the diffuse nuclear pattern. These data indicate that H3K9 tri-methyl is more consistent with heterochromatin and possibly gene silencing than H3 Lys9 di-methyl. We then performed double immuno-staining with these Abs on senescent IMR90s. Interestingly, H3K9 tri-methyl was enriched in the core of the SAHFs, while H3 Lys9 di-methyl stained whole area of the SAHFs (Fig. 2B). Furthermore, HP1 $\gamma$  was colocalized with H3K9 tri-methyl, indicating that HP1 could associate more preferentially with H3 Lys9 tri-methyl to create the higher ordered heterochromatic structure in senescence (Fig. 2C). Histone H3-Lys9 can be mono-, di-, or tri-methylated in vitro and in vivo. However, most available antibodies against H3-Lys9 methyl cannot discriminate these modifications. Furthermore, these anti-H3Lys9 methyl antibodies cross react with H3 Lys27 methyl, which is also involved in the gene silencing (10).

i) Generation of rabbit polyclonal anti-Lys9 tri-methyl Ab

KLH-conjugated polypeptide corresponding to amino acids 6-13 (TAR[tri-methyl-K]STGG-C) was used as an antigen. Anti-serum recognized both H3 Lys9 di- and tri-methyl. To obtain the H3 Lys9 tri-methyl specific Ab, anti-serum was first passed through di-methyl peptide column. Flowthrough of di-methyl peptide column then was applied to tri-methyl column, followed by elution with glycine (pH 2.5). Specificity to H3 Lys9 tri-methyl was confirmed by ELISA using non-modified H3 Lys9 peptide, H3 Lys9 di-, and tri-methyl peptides (Fig. 1).

ii) Generation of mouse monoclonal anti- Lys9 di-methyl Ab

We also tried to generate monoclonal Lys9-dimethyl Ab using the same polypeptide with di-methylated Lys9. One test serum specifically recognized the Lys9 di-methyl (Fig. 1). We have

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H3 Lys9 tri-methyl specific Ab immuno-staining showed punctuated pattern in MEFs. These H3 Lys9 tri-methyl enriched foci are clearly overlapped with DAPI-densely stained foci, which are pericentric heterochromatin (Fig. 2A). On the other hand, H3 Lys9 di-methyl Ab showed the diffuse nuclear pattern. These data indicate that H3K9 tri-methyl is more consistent with heterochromatin and possibly gene silencing than H3 Lys9 di-methyl. We then performed double immuno-staining with these Abs on senescent IMR90s. Interestingly, H3K9 tri-methyl was enriched in the core of the SAHFs, while H3 Lys9 di-methyl stained whole area of the SAHFs (Fig. 2B). Furthermore, HP1 $\gamma$  was colocalized with H3K9 tri-methyl, indicating that HP1 could associate preferentially with H3 Lys9 tri-methyl to create the higher ordered heterochromatic structure in senescence (Fig. 2C). Consistently, we showed higher RNA staining on the periphery of SAHFs (Fig. 2 in attached paper), although it is still unclear if H3 Lys9 di-methyl is important for the gene silencing or not. Consistently, we showed higher RNA staining in periphery of SAHFs (Fig. 2 in attached paper), although it is still unclear if H3 Lys9 di-methyl is important for the gene silencing or not.



## **Key Accomplishments**

- Senescent HDFs show characteristic chromatin structure, SAHFs.
  - SAHFs show heterochromatic features.
  - SAHFs show close correlation with E2F-target silencing.
  - E2F-targets are stably silenced in senescence.
  - SAHF-formation is dependent on the p16/Rb pathway.
  - Histone H3 Lys9 tri-methyl is strongly enriched in SAHFs.
- 
- Establishment of stable RNAi in HDFs using retroviral gene transfer system.
  - Generation of H3 Lys9 tri-methyl specific antibody.

## **Reportable Outcomes**

### **Paper (attached)**

Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence. *Cell*, 113(6):703-16, 2003

### **Meeting**

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe; SAHR; A NEW MARKER OF SENESENCE (Oral and poster). Era of Hope Department of Defense Breast Cancer Research Program Meeting, Florida, September 25-28, 2002

Masashi Narita, Masako Narita, Athena Lin, Stephen Hearn, Edith Heard, Scott Lowe; CHROMATIN REORGANIZATION CONTRIBUTES TO SENESENCE (oral). Molecular Genetics of Aging, CSHL, October 2-6, 2002

## Conclusion

We have found the novel type of heterochromatic structure (SAHFs) associated with senescence phenotype in human fibroblasts. Rearrangement of chromatin structure is strongly suggestive of the involvement of epigenetic regulation in senescence. SAHF-formation is dependent on the p16/Rb pathway, at least in part, because E1A and the knockdown of p16 or Rb by stable RNAi inhibit the SAHFs and p16 is sufficient to induce SAHFs. E2F-targets are stably silenced in senescence, which is consistent with the 'irreversibility' of the cell cycle arrest in senescence, and the silencing of these genes is closely correlated with SAHF-formation. Thus, p16/Rb-mediated epigenetic alterations would be crucial as tumor suppressor mechanism upon senescence. In the future, we will apply our system to p16-mediated mammary epithelial cellular senescence, focusing on the chromatin structure and gene expression pattern alterations.

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Figure 1

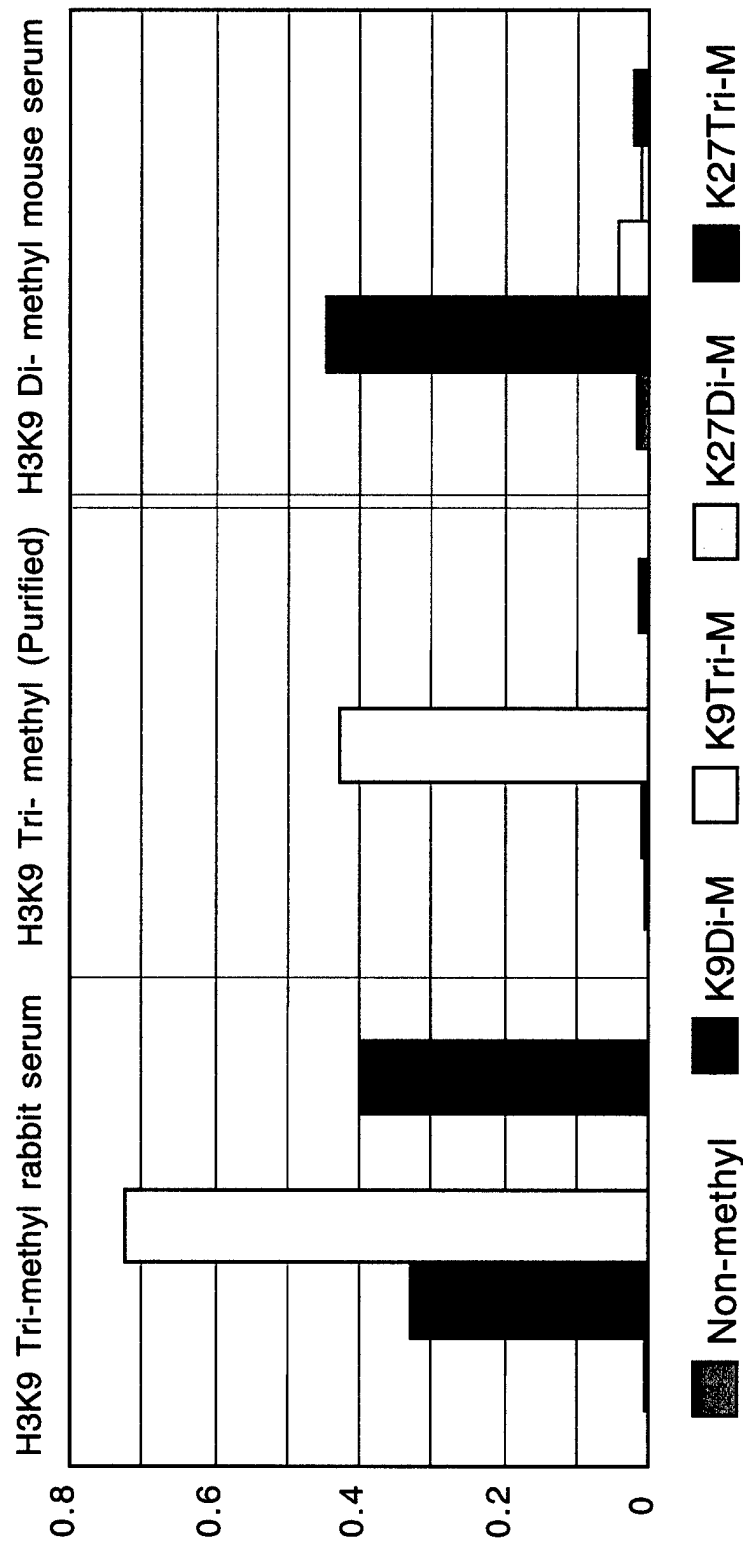
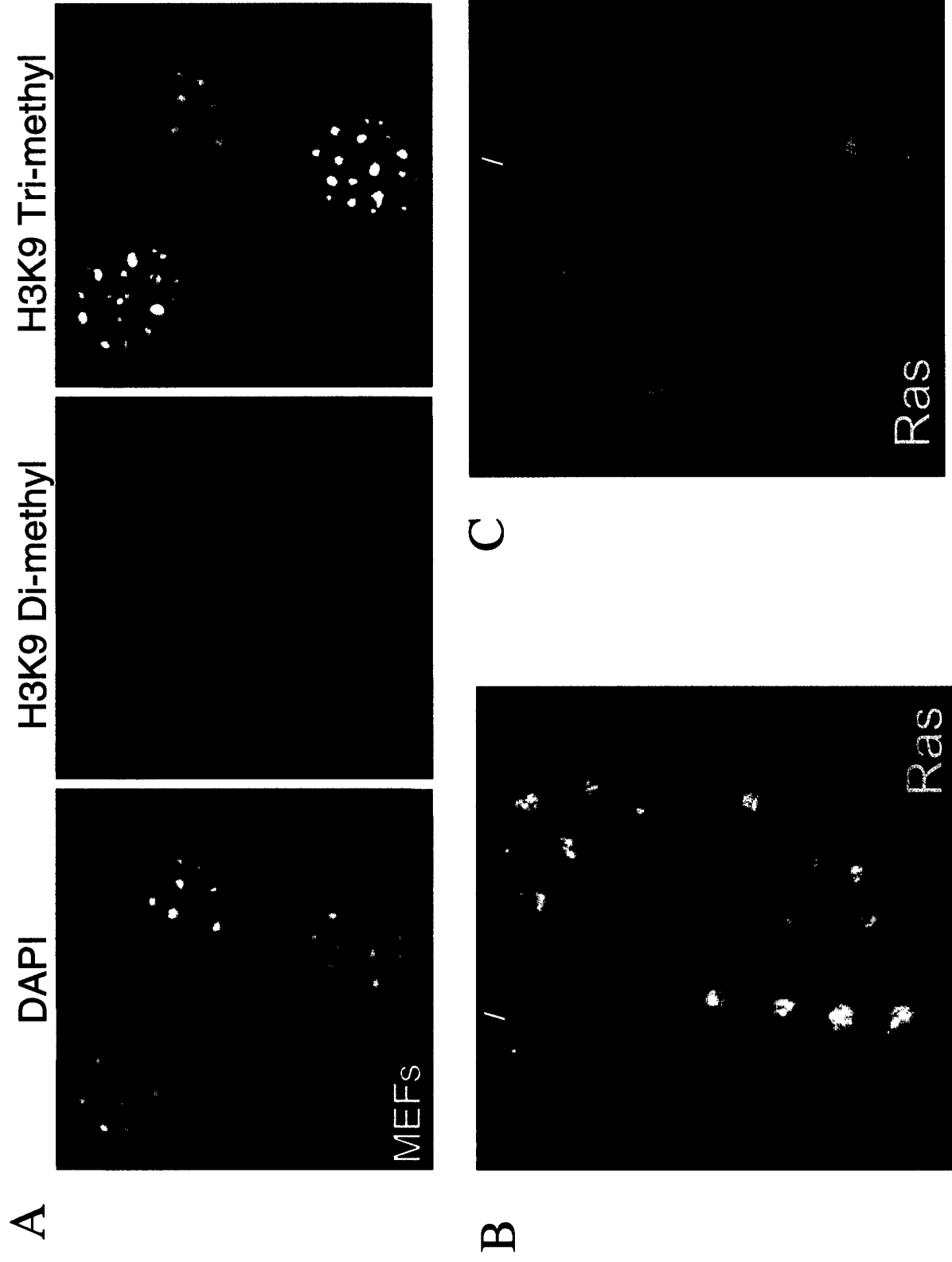


Figure 2



## **Figure Legends**

### **Figure 1. Specificity of new anti-histone H3K9 methyl Abs**

Affinity purified anti-histone H3K9 tri-methyl Ab (middle column) from crude serum (left column) shows no cross-reaction with non-modified, K9 di-methyl, and H3K27 methyl peptides by ELISA. Anti-H3K9 di-methyl mouse anti-serum shows high specificity to H3 di-methyl peptide (right column).

### **Figure 2. Different localization of histone H3K9 di- and tri-methyl in chromatin**

(A) NIH 3T3 cells were double stained with anti-histone H3K9 di- and tri-methyl Abs. Nuclei were counter stained with DAPI. Tri-methyl is colocalized with DAPI-deeply stained heterochromatin. (B) Ras senescent IMR90s were double stained with anti-histone H3K9 di- and tri-methyl Abs. Laser scanning confocal microscopy shows tri-methyl (red) is enriched in the core of the SAHFs, while di-methyl (green) shows broader pattern. (C) Ras senescent IMR90s were double stained with anti-HP1 $\gamma$  and histone H3K9 tri-methyl Abs. Laser scanning confocal microscopy shows HP1 $\gamma$  (green) is clearly colocalized with tri-methyl (red).

## Abstract

Molecular Genetics of Aging (CSHL)

October 2-6, 2002

### CHROMATIN REORGANIZATION CONTRIBUTES TO SENESCENCE

Masashi Narita, Masako Narita,, Athena Lin, Stephen Hearn, \*Edith Heard, Scott Lowe

The replicative exhaustion of Human diploid fibroblasts (HDFs) in culture is associated with an "irreversible" cell-cycle arrest known as replicative senescence. However, young cells can enter a state of cell-cycle arrest that is phenotypically identical to cellular senescence in response to expression of certain mitogenic oncogenes, oxidative stress, and DNA damage. Both replicative and 'premature' senescence involve activation of the p53 and p16/Rb tumor suppressor pathways. Here we describe a novel type of chromatin structure that has features of heterochromatin and appears to contribute to the senescence state. Specifically, we show that senescent HDFs harbor acquired regions of condensed chromatin that is enriched for HP1 proteins and histone H3 methylated on lysine 9. The appearance of these features requires the retinoblastoma gene product (Rb), since activation of Rb by enforced expression of p16 induces both senescence and chromatin alterations, and disruption of Rb function using viral oncoproteins prevents chromatin alterations in response to senescence-inducing stimuli. Interestingly, senescent cells display a defect in their ability to activate cell-cycle genes (relative to quiescence) in response to enforced expression of E2F, and disruption of Rb alleviates this repression. Moreover, enforced expression of SUV39H1, a histone H3 lysine 9 methyltransferase, induces premature senescence in HDFs in a manner that can be prevented by viral oncoproteins that target Rb. We propose that Rb-dependent changes in heterochromatic organization produce the specific gene expression patterns associated with senescence and, in particular, results in stable suppression of E2F target genes and a general non-responsiveness to mitogens.



## Abstract

Era of Hope

Department of Defense Breast Cancer Research Program Meeting

September 25-28, 2002

### SAHR; A NEW MARKER OF SENESCENCE

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe

There is ample evidence indicating that p53 plays a central role in tumor suppression in breast carcinogenesis. As a tumor suppressor, p53 is involved in both the apoptosis and senescence programs. Similar to apoptosis, various stimuli, including oncogenic ras and DNA damage, can induce a senescent phenotype, suggesting the existence of a common senescence machinery. The senescent phenotype continuously progress over the cell divisions, presumably without genetic alteration, which is strongly suggestive of epigenetic regulation in senescence program. While characteristic morphological changes in senescent cells are well known, the nuclear morphology of senescent cells is poorly characterized.

Human diploid fibroblasts, such as IMR90s, are well-established model system for cellular senescence. We have used this system along with retroviral gene transfer to characterize the nuclear morphology of senescent cells. IMR90s induced to senesce by Ras, as well as replicative senescent fibroblasts, show a characteristic DAPI staining pattern, which we have designated as SAHR, Senescence Associated Heterochromatic Regions. We have found a close correlation between SAHR formation and senescence markers such as, accumulation of p53/p16. Furthermore, the adenoviral oncoprotein E1A, which overrides senescence, also inhibits SAHR formation, and SAHRs are not found in quiescent cells. Taken together, these results indicate that SAHRs are a marker of senescence.

Immunofluorescence studies reveal that SAHRs colocalize with markers of heterochromatin, such as HP1 and Lys9 methylated histone H3. In contrast, Lys9/14 acetylation and Lys4 methylation of H3, which are consistent with euchromatin, are excluded from SAHRs. Electronmicrography confirms similar heterochromatic structures in senescent cells, where DNA is condensed and RNA is absent, suggesting that SAHRs are a novel type of heterochromatic structure. Given the fact that the levels of acetylated H3 are downregulated in senescent cells, our data suggest a global rearrangement of chromatin structure during senescence. If this is true, it could define a specific gene expression pattern particular to senescence and provide insights into an epigenetic regulation as a common machinery of senescence-related tumor suppression.

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### PUBLICATIONS

#### Original articles

Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence. *Cell*, 113(6):703-16, 2003

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# Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence

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## Summary

Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells and may act as a natural barrier to cancer progression. In this study, we describe a distinct heterochromatic structure that accumulates in senescent human fibroblasts, which we designated senescence-associated heterochromatic foci (SAHF). SAHF formation coincides with the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor to E2F-responsive promoters and is associated with the stable repression of E2F target genes. Notably, both SAHF formation and the silencing of E2F target genes depend on the integrity of the Rb pathway and do not occur in reversibly arrested cells. These results provide a molecular explanation for the stability of the senescent state, as well as new insights into the action of Rb as a tumor suppressor.

## Introduction

Cellular senescence was originally described as the process of cell cycle arrest that accompanies the exhaustion of replicative potential in cultured human fibroblasts (Hayflick, 1965). Senescent cells remain metabolically active; display characteristic changes in cell morphology, physiology, and gene expression; and typically upregulate a senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity (Campisi, 2001; Dimri et al., 1995; Shelton et al., 1999). Senescent cells are unable to express genes required for proliferation, even in a pro-mitogenic environment (Dimri et al., 1994, 1996). These features distinguish senescence from quiescence, a nonproliferative state that is readily reversed in response to mitogens. Although "replicative" senescence is triggered by telomere attrition, an identical endpoint (often

called "premature senescence" or "stasis") can be acutely produced in response to activated oncogenes, DNA damage, oxidative stress, and suboptimal cell culture conditions. These observations imply that cellular senescence, like apoptosis, is a cellular response to stress that limits the proliferation of damaged cells (Campisi, 2001; Mathon and Lloyd, 2001).

Although cellular senescence is typically studied in cultured cells, the process may be important in aging and cancer (Campisi, 2001). Cellular senescence is often considered a cellular counterpart of organismal aging and, indeed, increases in SA- $\beta$ -gal activity can be detected in cells from older individuals and patients with premature aging syndromes. Moreover, mutations that prevent DNA repair or promote chronic DNA damage can promote premature senescence in vitro and aging in vivo, and some genes that modulate senescence in cultured cells also affect lifespan in mice. Owing to its antiproliferative effects, senescence also appears to be a potent antitumor mechanism. Hence, mutations in certain tumor suppressor genes compromise senescence, thereby contributing to cell immortalization and cancer. Furthermore, cytotoxic agents used in cancer chemotherapy can induce cellular senescence, and defects in this process promote drug resistance in vivo (Chang et al., 1999; Schmitt et al., 2002; te Poele et al., 2002).

The Rb and p53 tumor suppressors are important senescence regulators. Rb and p53 are typically activated during senescence, and enforced expression of either protein induces senescence in some cell types (Ferbeyre et al., 2002; Lee et al., 2000). In human fibroblasts, DNA tumor virus oncoproteins that interfere with Rb and p53 function can bypass senescence. For example, SV40 large T antigen binds both Rb and p53 and overcomes replicative senescence, whereas large T mutants defective in binding either protein are less able to do so (Shay et al., 1991). Similarly, adenovirus E1A targets the Rb family and interferes with p53-mediated arrest and prevents senescence induced by oncogenic *ras* and DNA damaging agents (Lowe and Ruley, 1993; Serrano et al., 1997). In mouse embryo fibroblasts (MEFs), p53 loss is sufficient to overcome senescence, whereas inactivation of Rb alone has no obvious effect (Lowe and Sherr, 2003). Nevertheless, the Rb family contributes to senescence in this cell type, since cells lacking Rb along with the related p107 and p130 proteins fail to senesce in culture (Dannenberg et al., 2000; Sage et al., 2000).

In many instances, p53 and Rb are activated to promote senescence by products of the *INK4a/ARF* locus (Lowe and Sherr, 2003). This locus encodes two tumor suppressors, p16<sup>INK4a</sup> and p14<sup>ARF</sup> (p19<sup>ARF</sup> in mice), expressed from partially overlapping nucleotide sequences read in alternative reading frames. p16<sup>INK4a</sup> engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. In contrast, p14<sup>ARF</sup> increases the growth suppressive functions of p53 by interfering with its negative regulator, Mdm2. Both p16<sup>INK4a</sup> and p14<sup>ARF</sup> accumulate in senescent cells and can promote senescence when

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overexpressed (Lundberg et al., 2000). Moreover, mutations that affect *INK4a* or *ARF* can compromise senescence to varying degrees depending on species and cell type.

How Rb promotes senescence is not known. Rb family proteins are corepressors of the E2F transcription factors, and their combined activities are required for many aspects of cell cycle progression (Trimarchi and Lees, 2002). Rb-family members are thought to recruit histone deacetylases (HDACs) to E2F-dependant promoters, thereby deacetylating nearby histones and repressing gene expression. As cells approach S phase, cyclin D and E-dependent kinases phosphorylate Rb and free E2F, allowing it to act with histone acetyltransferases (HATs) to open chromatin structure and transactivate E2F-responsive genes important for G1 to S phase transition. The modification of histones by HATs and HDACs is dynamic and readily explains the reversibility of cell cycle arrest in quiescent cells. Recent studies suggest that p107 and p130, but not Rb, are associated with E2F-responsive genes during the cell cycle and quiescence (Rayman et al., 2002; Takahashi et al., 2000). Rb binds other proteins that influence gene expression, including the HP1 proteins and certain histone methyltransferases (Trimarchi and Lees, 2002). The relative contribution of these interactions to cell cycle regulation and Rb tumor suppressor functions has yet to be determined.

Although the molecular mechanisms underlying the irreversibility of cellular senescence remain poorly understood, these processes are extremely efficient. In fact, human fibroblasts almost never spontaneously escape replicative senescence and cannot be transformed unless the process is disabled (Campisi, 2001). Mutations that bypass cellular senescence can prevent the triggering event (e.g., telomerase activation) or act downstream of the damage signal to circumvent the senescence response (e.g., *INK4a* loss) (Brookes et al., 2002). Moreover, molecules that initiate senescence are often dispensable for its maintenance. For example, conditional expression of p53 and p16<sup>INK4a</sup> can efficiently induce senescence in some settings, and the cells remain arrested after removal of the respective gene (Dai and Enders, 2000; Ferbeyre et al., 2002; Sugrue et al., 1997). In this study, we investigate the molecular basis for the stability of the senescent state and show that this occurs, in part, through an Rb-directed process that involves alterations in heterochromatin and the stable silencing of E2F target genes. We propose that these processes contribute to the tumor-suppressive properties of the senescence program.

## Results

### Changes in Nuclear Morphology and Chromatin Structure Accompany Senescence

In IMR90 human diploid fibroblasts undergoing senescence, we observed a characteristic nuclear morphology involving changes to the nucleolus and the organization of DNA. IMR90 cells induced to senesce by exogenous expression of oncogenic Ras, expression of activated MEK, treatment with the chemotherapeutic drug etoposide, enforced expression of p16<sup>INK4a</sup>, or extensive passaging (i.e., replicative senescence), typi-

cally displayed one large nucleolus and punctate DNA foci as visualized by DAPI staining (Figure 1A; data not shown). In contrast, exponentially growing IMR90 cells, as well as cells made quiescent by serum withdrawal or confluence, usually displayed several small nucleoli and a more uniform DAPI staining pattern (Figure 1A; data not shown). IMR90 cells expressing E1A, which readily escape senescence, also did not develop pronounced nucleoli or DNA foci in response to Ras (Figure 1A). Finally, senescent WI38 cells, another normal fibroblast strain, acquired prominent DNA foci that appeared indistinguishable from senescent IMR90 cells (data not shown). Therefore, the changes to nuclear architecture are not unique to IMR90 cells, nor are they a necessary consequence of cell cycle arrest or oncogenic *ras* expression. Rather, these changes are specific for the senescent state.

In order to determine the kinetics of the appearance of senescence-associated DNA foci, we focused on the effects of oncogenic *ras*, as it acutely and reproducibly induces senescence in IMR90 cells over several days (Serrano et al., 1997). IMR90 cells were infected with retroviruses coexpressing oncogenic *ras* and a selectable marker (Figure 1B). After a brief drug selection to eliminate uninfected cells, cell populations were analyzed at various times for p16<sup>INK4a</sup> and Rb expression, cellular proliferation (BrdU incorporation), the onset of senescence (SA- $\beta$ -gal staining), and the appearance of DNA foci (DAPI staining). Cells expressing oncogenic *ras* accumulated p16<sup>INK4a</sup> and hypophosphorylated Rb between 3 and 5 days postselection (Figure 1C), a time when these cells stopped incorporating BrdU and became positive for SA- $\beta$ -gal (Figure 1D). Although control cells continued to proliferate and did not display senescence-related changes, the appearance of DNA foci in *ras*-expressing cells coincided precisely with cell cycle exit and the onset of senescence. Moreover, BrdU-positive nuclei containing DNA foci were never observed, implying that DNA synthesis and DNA focus formation were mutually exclusive processes.

### Senescence-Associated DNA Foci Do Not Contain Active Sites of Transcription

To further characterize the nuclear changes that accompany senescence, normal and senescent IMR90 cells were examined by electron microscopy. In contrast to exponentially growing or quiescent controls (Figure 2A; data not shown), senescent nuclei showed prominent nucleoli, an irregular nuclear envelope, and electron-dense regions interspersed throughout the nucleoplasm (Figure 2B). To test whether these electron-dense regions correspond to the DNA foci observed by DAPI staining, we immunolabeled quiescent and senescent cells with an anti-DNA antibody and a secondary antibody conjugated to colloidal gold. In contrast to the diffuse pattern observed in quiescent cells, DNA labeling was concentrated in the electron-dense regions of senescent nuclei (Figure 2, compare C and D).

We also examined the relationship between DNA foci and sites of transcription. To assess global RNA localization, senescent cells were labeled with gold-conjugated RNase, which binds to and localizes RNA following electron microscopy. RNA was mostly excluded from the

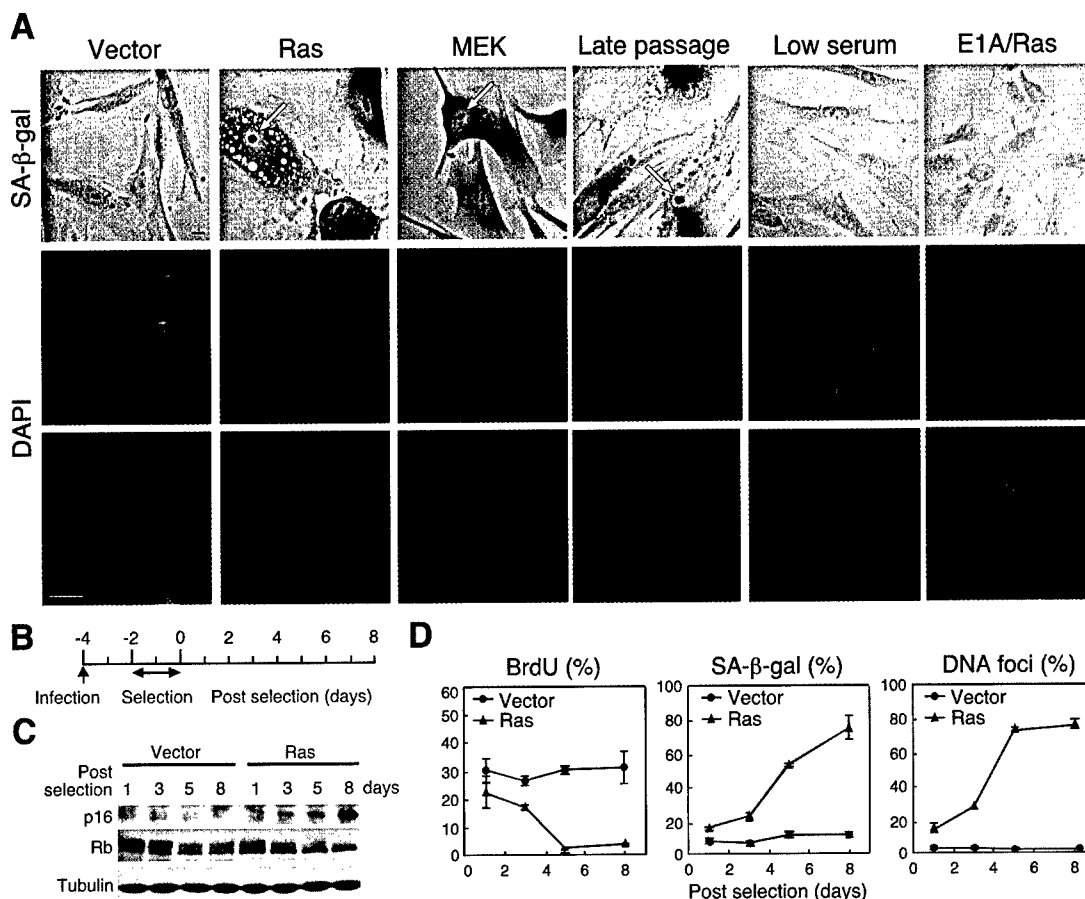


Figure 1. DNA Foci Accumulate in Senescent Cells

(A) IMR90 cells containing empty vector, H-rasV12 (Ras), MEK1 Q56P (MEK), or E1A12S/H-rasV12 (E1A/Ras) were stained for SA-β-gal activity, a classical marker of senescence, followed by DAPI staining, 6 days postselection. Late passage and quiescent cells, induced by culture in 0.1% serum for 48 hr (Low serum), are also shown for comparison. Enlarged images of DAPI staining are shown in the lower panels. Arrows indicate prominent nucleoli. Scale bars are equal to 10 μm.

(B) Experimental design and time frame. For cells that were serially selected, day zero is set after the first selection.

(C) Protein expression of p16<sup>INK4a</sup> and Rb was assessed by Western blotting using lysates from cells containing empty vector or H-rasV12 (Ras) over a period of days. Tubulin serves as a loading control.

(D) IMR90 cells containing empty vector or H-rasV12 (Ras) were scored for percentage of BrdU incorporation (left image), SA-β-gal activity (middle image) and the presence of DNA foci (right image), at the indicated days post selection.

interior of the large DNA foci and was instead interspersed throughout the nucleoplasm and concentrated in small foci (Figure 2D). The latter structures may correspond to perichromatin fibrils or RNA-containing nuclear bodies, known to be active transcription sites (Spector, 2001). To localize specific gene transcription, we performed RNA fluorescence in situ hybridization (RNA FISH) using bacterial artificial chromosome (BAC) clones harboring the *cyclin A* and *INK4a* genes as probes. *Cyclin A* is expressed in dividing cells and down-regulated during senescence. As expected, two *cyclin A* signals were observed in exponentially growing cells (Figure 2E, vector). In cells undergoing senescence, these two signals were often observed at the periphery of DNA foci and subsequently extinguished (Figure 2E, compare Ras PS1 to Ras PS7). In contrast, *INK4a* is silent in dividing cells but upregulated during senescence. Concordantly, no *INK4a* RNA FISH signal was observed in growing cells, while two signals appeared in most senescent cells (Figure 2E, compare vector to

Ras PS7). In all cases, the positive signals were either at the periphery or outside of the DNA foci. Hence, senescence-associated DNA foci are condensed regions of DNA that correlate with transcriptionally inactive sites.

#### Senescence-Associated DNA Foci Have Features of Heterochromatin

The senescence-associated DNA foci are reminiscent of heterochromatin, which encompasses transcriptionally inactive regions of the genome that are packaged into highly dense chromatin fibers during interphase. Heterochromatin is important for various nuclear functions, including chromosome segregation, nuclear organization, and gene silencing (Henikoff, 2000; Jenuwein, 2001). At the molecular level, heterochromatic regions often lack histone H3 that is acetylated on lysine 9 (K9Ac-H3) and methylated on lysine 4 (K4M-H3). In contrast, these regions are usually enriched for histone H3 methylated on lysine 9 (K9M-H3). Notably, methylated lysine 9 provides a docking site for HP1 proteins (Ban-

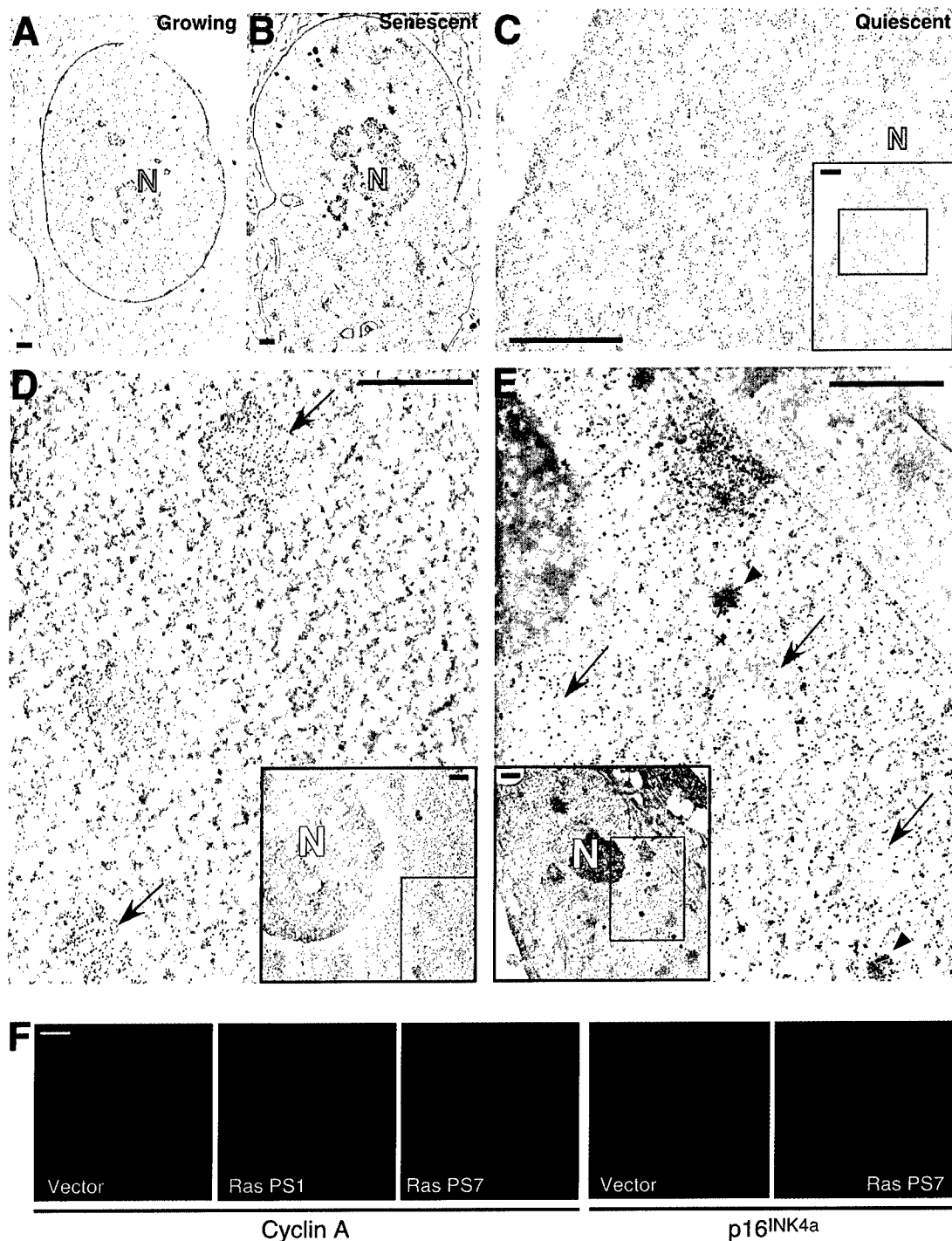


Figure 2. Senescence-Associated DNA Foci Are Not Sites of Active Transcription

(A and B) Electron microscopy images of vector control (Growing) (A) and Ras-senescent IMR90 cells (senescent) (B) "N" is nucleolus. Scale bars are equal to 1  $\mu$ m.

(C) High magnification of a nucleus from low serum quiescent cells showing DNA detected using a monoclonal anti-DNA antibody and gold-coupled secondary antibody. Scale bar is equal to 1  $\mu$ m.

(D) Higher magnification of a senescent nucleus (from rectangle in inset) showing DNA detected using a monoclonal anti-DNA antibody and gold-coupled secondary antibody. Arrows show the corresponding higher electron density regions of (B). Scale bar is equal to 1  $\mu$ m.

(E) Senescent nucleus showing RNA labeled with gold-coupled RNase T1 at same magnification as (D). Arrows show electron-dense regions corresponding to DNA foci in (B). RNA-rich small foci are likely to represent perichromatin fibrils or RNA-containing nuclear bodies (arrowheads). Scale bar is equal to 1  $\mu$ m.

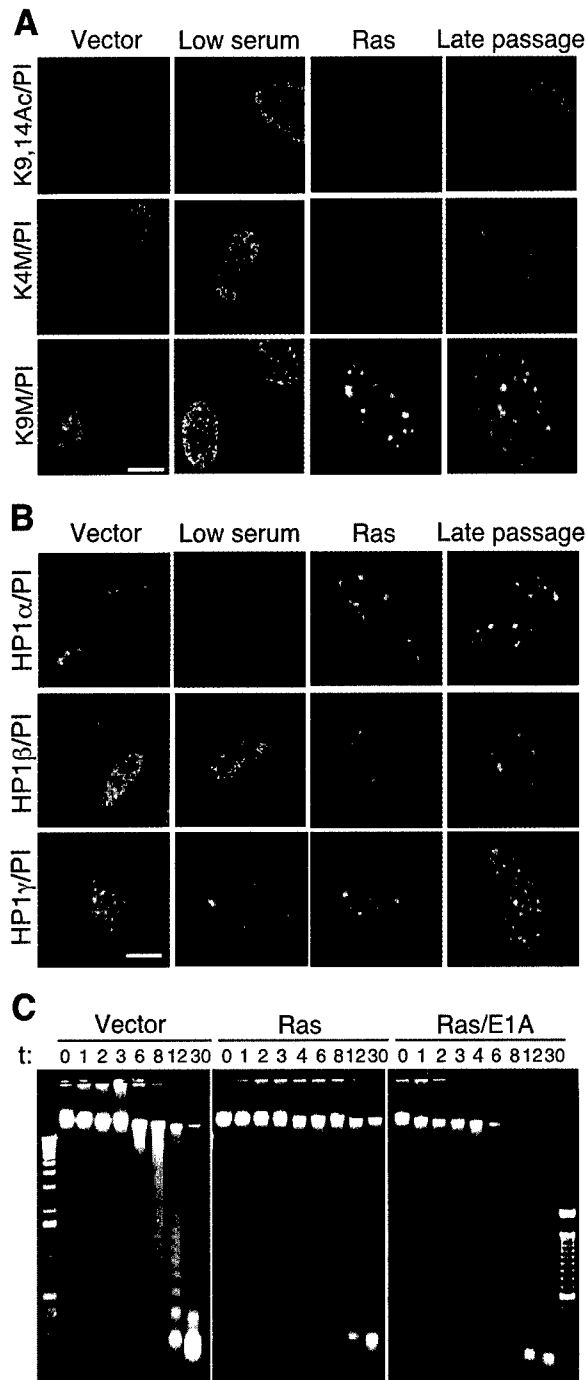
(F) RNA FISH for *Cyclin A* and *INK4a* (*p16<sup>INK4a</sup>*) was performed on vector control and Ras-senescent IMR90 cells (Ras) at the indicated post selection (PS) days. Intense signals of *INK4a* in Ras-senescent cells were localized at the periphery or outside of the DNA foci. DNA was counterstained by DAPI, which was pseudocolored green. Scale bar is equal to 5  $\mu$ m.



nister et al., 2001; Lachner et al., 2001), a family of adaptor molecules that are required for heterochromatin assembly and are involved in epigenetic gene regulation.

To determine whether the senescence-associated DNA foci are related to heterochromatin, we conducted confocal fluorescence microscopy on growing, quiescent, and senescent cells using modification-specific antibodies against histone H3 or the HP1 proteins  $\alpha$ ,  $\beta$ , and  $\gamma$ . Growing and quiescent cells expressed histones with all modifications examined, and these appeared distributed throughout the nucleoplasm (Figure 3A, vector and low serum). In marked contrast, senescent cells showed a more distinctive localization of modified histones. Consistent with their preference for euchromatic regions, K9Ac-H3 and K4M-H3 were largely excluded from DNA foci. Conversely, K9M-H3 was concentrated in the DNA foci of senescent cells (Figure 3A, Ras and Late passage). Similarly, all three HP1 proteins were dispersed throughout the nucleoplasm in normal and quiescent cells but were concentrated to varying degrees in the DNA foci of senescent cells (Figure 3B). This distinctive pattern was not due to variable antibody accessibility, since IMR90 cells expressing a green fluorescence protein (GFP)-HP1 $\beta$  fusion protein also showed a GFP pattern that was dispersed in normal cells but concentrated in DNA foci of senescent cells (Supplemental Figure S1A available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Consequently, senescence-associated DNA foci contain heterochromatin and were defined as "senescence-associated heterochromatic foci" (SAHF).

Constitutive heterochromatin consists of large regions of heterochromatin found near the centromeres and telomeres of mammalian chromosomes during interphase. In principle, the SAHFs might result from a redistribution of preexisting heterochromatin (e.g., owing to changes in nuclear structure) rather than from new heterochromatin that accumulates in senescent cells. However, several observations suggest that this is unlikely. First, no substantial colocalization was observed between SAHF formation and either centromeres or telomeres, as determined by immunofluorescence using antiserum that recognizes centromeres or an antibody directed against telomere associated protein TRF2, respectively (Supplemental Figure S1B available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Second, senescent cells (induced by oncogenic *ras*) displayed a substantial increase in the total amount of chromatin-bound HP1 $\beta$  and HP1 $\gamma$  relative to normal proliferating and quiescent cells (Supplemental Figure S1C available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>). Importantly, E1A abolished the accumulation of chromatin-bound HP1 in response to oncogenic *ras* expression, indicating that these changes were linked to the senescent state. Finally, consistent with the known resistance of heterochromatin to limited nuclease digestion (Leuba, et al., 1994), DNA from senescent cells was more resistant to micrococcal nuclease compared to normal cells and E1A-expressing cells that had bypassed *ras*-induced senescence (Figure 3C). Together, these results imply that formation of a distinct heterochromatic structure accompanies the senescence process.



**Figure 3. Senescent Cells Accumulate Features of Heterochromatin**  
(A) Confocal images of indirect immunofluorescence of acetylated histone H3 on Lysine 9/14 (K9/14Ac), methylation on lysine 4 (K4M), and methylation on lysine 9 (K9M) (green) in normal growing (vector), low serum quiescent (low serum), H-rasV12 (Ras) senescent, or replicative senescent IMR90 cells (late passage). The DNA was counterstained by propidium iodide (PI) (red). Merged images are shown.

(B) Localization of endogenous HP1 proteins (green) was determined by indirect immunofluorescence using the indicated antibodies in the cells described in (A). DNA was stained with propidium iodide (PI) (red). Merged images are shown.

(C) Micrococcal nuclease digestion of detergent-permeabilized cells from growing (Vector), senescent (Ras), and Ras/E1A transformed cells. DNA was isolated from cells after digestion for the indicated time (min) and subjected to agarose gel electrophoresis.

### Some E2F Target Promoters Acquire Heterochromatic Features during Senescence

The changes in euchromatic and heterochromatic organization that accompany SAHF formation might produce senescence-specific changes in gene expression. Since heterochromatin has been linked to gene silencing, we reasoned that SAHFs might contribute to the stable cell cycle arrest that is a hallmark of senescence. If true, then components of euchromatin or heterochromatin would be associated with genes that are induced or repressed during senescence, respectively. To test this, we examined the association of K9/14Ac-H3, K9M-H3, and HP1 $\gamma$  to the promoters of several genes in senescent cells in vivo using chromatin immunoprecipitation. We focused on E2F-target promoters because these genes are essential for cell cycle progression and are negatively regulated by the Rb family which, in turn, influences senescence. These genes are also constitutively induced by E1A, which inactivates Rb and prevents SAHF formation and senescence. Here, we analyzed senescent cells produced by oncogenic *ras*, because these cells undergo a relatively synchronous arrest.

We first examined the binding of K9/14Ac-H3, a marker of euchromatin, for its presence on genes associated with senescence. *Stromelysin-1* and *INK4a* are upregulated during senescence, whereas *cyclin A* and *PCNA* are E2F-target genes that are repressed. Compared to normal cells, the amount of K9/14Ac-H3 bound to the *stromelysin-1* promoter increased in senescent cells, although no change was observed on the *INK4a* promoter (Figure 4A, compare lanes 2 and 3). In contrast, the amount of K9/14Ac-H3 that associated with *cyclin A* and *PCNA* declined during senescence. Interestingly, E1A abolished the increase in K9/14Ac-H3 binding to the *stromelysin-1* promoter, but produced an increase in K9/14Ac-H3 association with the *cyclin A* and *INK4a* promoters (Figure 4A, compare lanes 3 and 4). These latter results are consistent with the known E2F responsiveness of *cyclin A*, and the observation that *INK4a* levels often increase following Rb inactivation (Stott et al., 1998). The amount of K9/14Ac-H3 bound to the *stromelysin-1* promoter did not increase in quiescent cells (produced by serum depletion or confluence), indicating that this effect was specific for senescence (Figure 4B, compare lane 2 to lanes 3 and 4). However, the amount of K9/14Ac-H3 bound to the *cyclin A* and *PCNA* promoters also declined in quiescent cells, indicating that hypoacetylation of histones on E2F target promoters is not unique to senescence.

We next examined the occupancy of E2F target promoters by K9M-H3 and HP1 $\gamma$ : two proteins that are enriched in the SAHFs and are known to be involved in heterochromatin formation (Bannister et al., 2001; Lachner et al., 2001). In marked contrast to acetylated histone H3, the amount K9M-H3 associated with the *cyclin A* and *PCNA* promoters increased in senescent cells relative to quiescent cells (Figure 4C, compare lanes 1 and 2). Although the amount of K9M-H3 detected from experiment to experiment was variable, similar results were produced with two anti-K9M-H3 antibodies. Similarly, HP1 $\gamma$ , which binds K9M-H3 in the context of heterochromatin, also associated with the *PCNA* and *cyclin A* promoters in senescent but not quiescent cells (Figure 4C, compare lanes 3 and 4). Importantly, E1A,

which prevents senescence and SAHF formation, also prevented *ras*-induced HP1 $\gamma$  and K9M-H3 accumulation on the two E2F target promoters (Figure 4D, compare lanes 1–2 and 7–8). Therefore, the accumulation of K9M H3 and HP1 $\gamma$  on the promoters examined is not detected when cells exit the cell cycle into a reversible quiescent state, but accompanies the more stable senescence-like arrest.

### Rb Associates with E2F-Responsive Promoters in Senescent but Not Quiescent Cells

Rb is an important regulator of E2F-responsive genes and contributes to cellular senescence (Campisi, 2001). Although Rb is often linked to normal cell cycle progression, recent reports indicate that p107 and p130 are the predominant Rb family members bound to E2F responsive promoters in quiescent and G1 cells (Rayman et al., 2002; Takahashi et al., 2000). Interestingly, in addition to the E2Fs, Rb can also associate with HP1 and histone methyltransferases such as SUV39H1, raising the possibility that Rb helps direct the process of histone methylation and HP1 recruitment to E2F responsive promoters during senescence. Consistent with this possibility, Rb showed a limited colocalization with SAHFs in the nuclei of senescent cells, which was greater than that observed for p107 and p130 (Supplemental Figure S2 available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>).

To determine whether Rb might occupy E2F target gene promoters during senescence, we examined the association of all three pocket proteins with the *cyclin A* and *PCNA* promoters in quiescent (confluent) and senescent (*ras*) cells using chromatin immunoprecipitation. As expected, p107 and p130 were readily detected on the *cyclin A* and *PCNA* promoters in quiescent cells (Figure 4E, lanes 1 and 3). While the association between p107 and these promoters typically declined in senescent cells, p130 was retained to varying degrees (Figure 4E, lanes 2 and 4; data not shown). In contrast, Rb was difficult to detect on the *cyclin A* and *PCNA* promoters in quiescent cells, but was detected on these promoters in senescent cells (Figure 4E, compare lanes 5 and 6). Although the Rb signal in senescent cells was often weak (perhaps owing to antibody inaccessibility to the Rb localized to compacted chromatin), we observed similar results in multiple experiments (data not shown). These results indicate that Rb can physically associate with some E2F target promoters in senescent cells and raise the possibility that Rb plays a special role in this process.

### E2F-Responsive Genes Are Stably Repressed in Senescent Cells

In principle, the altered chromatin state accompanying SAHF formation and the binding of K9M-H3, HP1, and Rb to E2F responsive promoters could stably silence the expression of E2F responsive genes and produce a permanent insensitivity to mitogenic signals. Indeed, in contrast to quiescent cells, mitogenic growth factors are unable to activate E2F target genes in senescent cells (Good et al., 1996). Furthermore, consistent with their incorporation into a more condensed chromatin state, we have observed that some E2F target genes are more resistant to MNase digestion in senescent compared to quiescent cells (see Supplemental Figure

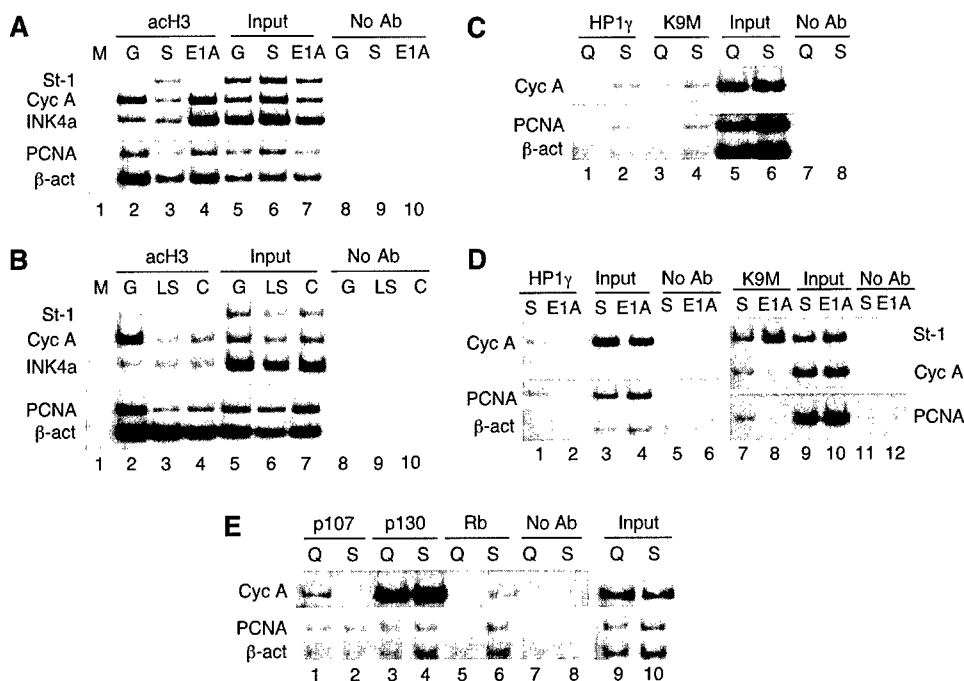


Figure 4. Chromatin Immunoprecipitation Analysis of E2F Target Promoters in Quiescent and Senescent Cells

(A and B) Chromatin immunoprecipitation assay (ChIP) using either an antibody against acetylated histone H3 (acH3), no antibody (No Ab) or nuclear extract (input). DNA fragments were amplified by PCR from the promoter regions of *stromelysin-1* (St-1), *cyclin A* (Cyc A), *INK4a*, *PCNA*, and  $\beta$ -actin ( $\beta$ -act). Buffer without nuclear extract served as Mock (M) control. Normal growing IMR90 cells (G), Ras-senescent cells (S), and E1A/Ras transformed cells (E1A) were used in (A); normal growing cells (G), low serum (LS), and confluent quiescent cells (C) were used in (B). DNA fragments were amplified by PCR from the promoter regions of *cyclin A* (Cyc A), *PCNA*, and  $\beta$ -actin ( $\beta$ -act). (C and D) ChIP assays were performed using HP1 $\gamma$  and K9M-H3 antibodies on extracts from quiescent (Q), Ras-senescent (S), and E1A/Ras-expressing (E1A) IMR90 cells.

(E) ChIP assays were performed as in (C) using p107, p130, or Rb antibodies on extracts from quiescent (Q) and Ras-senescent (S) cells.

S3 available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>. To examine this insensitivity directly, we introduced E2F-1 into quiescent (serum-starved) or senescent (ras) cells using adenovirus-mediated gene transfer and examined the expression of several E2F target genes using quantitative real-time RT-PCR 48 hr after infection. This approach allowed us to circumvent the mitogenic signaling pathways that are otherwise required for E2F activation.

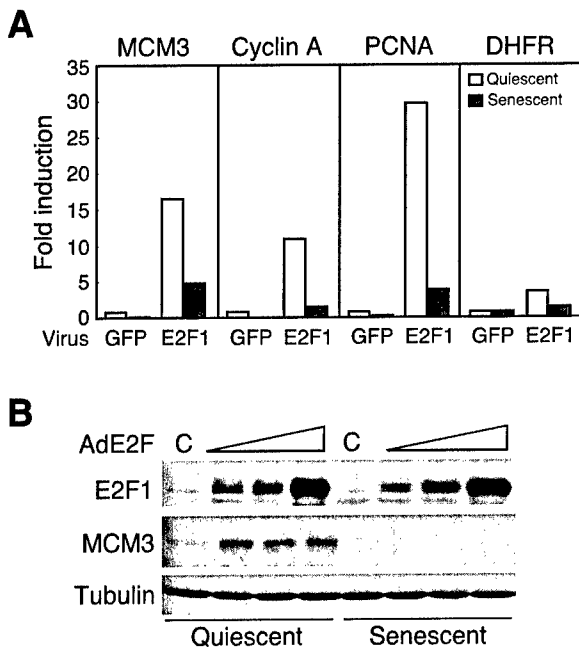
Consistent with previous reports (Leone et al., 1998), expression of E2F-1 in quiescent cells produced a substantial increase in *MCM3* and other E2F-targets, *cyclin A*, *PCNA*, and *DHFR* expression (Figure 5A). This effect was a result of E2F-1 and not adenovirus infection, since a control adenovirus did not induce these E2F targets. In contrast, E2F-1 was unable to effectively induce E2F target genes in senescent cells (Figure 5A), despite similar levels of E2F-1 expression (Figure 5B; data not shown). Although some E2F target genes could be induced in senescent cells at high concentrations of E2F-1 (data not shown), their relative insensitivity to E2F-1 was observed over a substantial concentration range (Figure 5B). Therefore, E2F target genes are stably repressed in senescent cells.

#### The p16<sup>INK4a</sup>/Rb Pathway Is Required for SAHF Formation and E2F-Target Silencing

The potential role of Rb in directing SAHF formation and the silencing of E2F target genes may explain, in part,

its role in controlling senescence. The E1A oncoprotein, which can contribute to cellular immortalization, counters Rb function and prevents SAHF formation (see Figure 1A). Interestingly, Rb interacts with HP1 proteins via its "LXCXE" motif, and this interaction can be disrupted by the E1A oncoprotein (data not shown). To further characterize the impact of the Rb family on SAHF formation and E2F target gene silencing, we examined the ability of E1A to affect cellular proliferation (BrdU incorporation), SA- $\beta$ -gal activity, SAHF formation, and the expression of E2F target genes in response to oncogenic *ras*. In some experiments, we replaced oncogenic *ras* with p16<sup>INK4a</sup>, which engages the Rb pathway and is sufficient to induce senescence in normal diploid fibroblasts (Brookes et al., 2002).

As expected, full-length E1A abrogated *ras*-induced senescence (Figure 6). Hence, E1A-expressing cells continued to proliferate, did not accumulate SA- $\beta$ -gal activity or SAHFs, and expressed high levels of E2F-responsive genes (Figure 6C, see also Figure 1A). Interestingly, E1A  $\Delta$ N, which preserves the LXCXE motif and targets the Rb family but not the p300 and p400 proteins, was not as effective as full-length E1A at preventing cell cycle arrest or SA- $\beta$ -gal accumulation (Figures 6A and 6B), but retained its ability to prevent SAHF formation and repression of E2F targets (Figures 6B and 6C). Enforced expression of p16<sup>INK4a</sup> also induced SA- $\beta$ -gal activity, SAHF formation, and repressed E2F target genes and, in this setting, E1A  $\Delta$ N was able to completely reverse the phenotype (Figures 6A and 6B). Finally, cells



### Figure 5. E2F Target Genes in Senescent Cells Are Resistant to Activation by E2F

(A) Quiescent (low serum) and Ras-senescent IMR90 cells were infected with adenoviruses expressing GFP and E2F-1. MCM3, Cyclin A, PCNA, and DHFR levels were determined by real-time RT-PCR.

(B) Quiescent (by confluence) and Ras-senescent IMR90 cells were infected with adenoviruses expressing GFP and E2F-1. MCM3 level was determined by Western blotting (triangle indicates increasing multiplicities of infection).

expressing a dominant-negative p53 underwent cell cycle arrest, accumulated SAHF, and downregulated E2F targets (Figure 6), indicating that these aspects of the senescent program can occur independently of p53. Therefore, activation of one or more Rb-family proteins is necessary, and perhaps sufficient, for SAHF formation and the stable repression of E2F target genes.

The fact that Rb preferentially associates with specific E2F target promoters during senescence suggests that it may play a particularly important role in SAHF formation and the silencing of E2F target promoters. Although the E1A data are consistent with this view, E1A also binds p107 and p130, and these proteins can also associate with E2F target promoters in senescent cells. To directly target Rb, we exploited stable RNA interference (RNAi) technology to specifically knock down p16<sup>INK4a</sup> and Rb expression. A series of short hairpin RNAs (shRNAs) targeting different sequences in the p16 and Rb mRNAs were generated that had  $\geq 6$  nucleotide differences from any other known human gene, and several were identified that efficiently suppressed p16<sup>INK4a</sup> or Rb expression (data not shown). Next, IMR90 cells were infected with retroviruses expressing an shRNA directed against p16<sup>INK4a</sup> (sh-p16) or Rb (sh-Rb), in combination with a *ras*-expressing retrovirus to induce senescence. Both p16<sup>INK4a</sup> and Rb were stably repressed by their respective shRNA relative to the control vector (Figure 7A, compare lanes 4 to 5 for p16; compare lanes 4–6 for Rb). Interestingly, like E1A  $\Delta N$ , neither sh-p16 nor sh-

Rb prevented eventual cell cycle exit, but both reduced SAHF formation (Figure 7B) and prevented the silencing of E2F targets (Figure 7A, compare lane 4–5 and 6 for MCM3, cyclin A, and PCNA).

These effects were more dramatic when examined at the single-cell level (Figure 7C). As expected, most cells coexpressing *ras* with a control shRNA vector contained SAHFs, as assessed by DNA foci and punctate HP1 $\gamma$  staining. These cells also expressed low levels of MCM3, which is an E2F-regulated gene, critical for the initiation of DNA replication. In contrast, cells coexpressing E1A  $\Delta$ N did not produce SAHFs and expressed extremely high MCM3 levels. Cells coexpressing sh-p16 or sh-Rb were more heterogeneous; whereas many cells did not form SAHFs and expressed high MCM3 levels, a subset developed SAHFs and silenced MCM3. Hence, a close correlation exists between SAHF formation and the repression of the E2F target genes examined, which is consistent with a stable silencing of these genes. These data demonstrate that Rb controls heterochromatin structure and gene silencing in senescent cells and provide an explanation for the stability of the senescent state.

## Discussion

Cellular senescence is considered a permanent form of cell cycle arrest that is characterized by distinct changes in gene expression and an extreme insensitivity to mitogenic stimuli. We show that senescent IMR90 human fibroblasts accumulate a distinct chromatin structure enriched with heterochromatin proteins (designated SAHF) that excludes active transcription and is characterized by the accumulation of K9M-H3 and HP1 proteins. We further show that heterochromatin-associated proteins and the Rb tumor suppressor can accumulate on the E2F-responsive promoters in senescent but not quiescent cells, and that these changes are associated with more stable repression of E2F responsive genes. Notably, Rb is required for SAHF formation and E2F target gene silencing. We suggest that Rb-directed changes in heterochromatin organization contribute to senescence-associated changes in gene expression and the permanence of the senescent state.

## Senescent Cells Accumulate a Distinctive Type of Heterochromatin

The organization of DNA into heterochromatin contributes to nuclear organization, chromosome structure, and gene silencing (Dillon and Festenstein, 2002; Lachner and Jenuwein, 2002). Constitutive heterochromatin primarily encompasses the pericentric regions of chromosomes and is important for chromosome segregation and the silencing of repetitive elements. Facultative heterochromatin is developmentally controlled and contributes to gene regulation during differentiation and influences dosage compensation. Here, we identify a distinctive type of facultative heterochromatin—designated SAHF—that accumulates in senescent cells. SAHFs are observed in interphase nuclei and contain the heterochromatin-associated proteins K9M-H3 and HP1, exclude histone modifications found in euchromatin (e.g., K9Ac-H3 and K4M-H3), and are not sites of

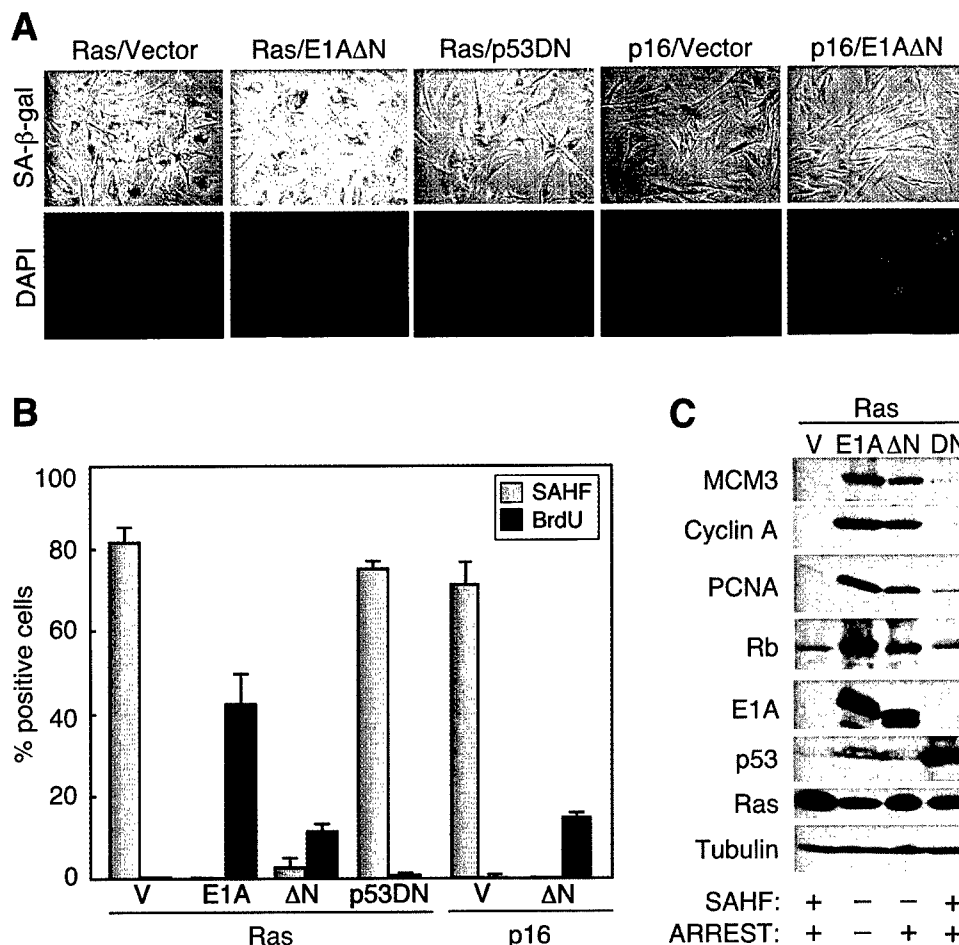


Figure 6. Inactivation of the Rb Family Proteins by E1A Prevents SAHF Formation and Gene Silencing

(A) IMR90 cells expressing H-rasV12 (Ras) in combination with either vector (vector), *E1AΔN* (lacking 2–36 amino acids), or *p53<sup>175H</sup>* (*p53DN*), and IMR90 cells expressing *p16<sup>INK4a</sup>* in combination with either vector or *E1AΔN* were stained for SA-β-gal activity at day 6 (see Figure 1B). DAPI staining is shown below.

(B) Same cells as (A), as well as IMR90 cells expressing both *E1A* and H-rasV12 were assessed for SAHF formation and BrdU incorporation at the same time point as (A).

(C) The expression of the indicated protein was determined by immunoblotting using extracts from IMR90 cells expressing H-rasV12 (Ras) in combination with either empty vector (V), *E1A*, or *E1AΔN* (ΔN), a *p53* dominant-negative (DN) at the same time point as in (A) and (B).

active transcription. SAHFs are distinct from pericentric heterochromatin, and their appearance is accompanied by an increase in HP1 incorporation into senescent chromatin and an enhanced resistance of senescent DNA to nuclease digestion.

SAHF formation requires an intact Rb pathway, since expression of *E1A*, or inactivation of either *p16<sup>INK4a</sup>* or *Rb*, can prevent their appearance. During the initial phases of senescence, *Rb* might control the nucleation of heterochromatin at specific sites throughout the genome, which then spreads by the action of histone methyltransferases and recruitment of HP1 proteins. HP1 proteins have the capacity to dimerize and may interact to form higher order chromatin structures once a critical mass has been reached (Brasher et al., 2000; Nielsen et al., 2001a). A similar pattern of nucleation and spreading occurs during silencing of the mating type locus in *S. pombe*, position effect variegation in *Drosophila*, and X inactivation in mammalian cells, although HP1 proteins do not accumulate on the inactive X (Heard et al., 2001;

Peters et al., 2002). Importantly, SAHF formation correlates precisely with cell cycle exit and the silencing of E2F target genes.

Our results suggest that SAHFs causally contribute to cellular senescence, at least in part, by controlling the stability of the arrest. In this study we characterized the formation of SAHFs in IMR90 human diploid fibroblasts, a cell type where cellular senescence has been studied extensively. Interestingly, SAHF formation also occurred in senescent WI38 human fibroblasts, but was less pronounced in senescent BJ human fibroblasts and MEFs (data not shown). These differences are noteworthy in light of recent reports showing that senescent BJ fibroblasts and MEFs can be stimulated to divide upon disruption of the *p53* pathway, whereas WI38 cells cannot (Dirac and Bernards, 2003; Beauséjour et al., submitted). Furthermore, senescent IMR90 cells do not divide following the introduction of *E1A*, although quiescent IMR90 cells readily do so (S.N. and S.W.L., unpublished). Together, these results link SAHFs to an essentially irre-

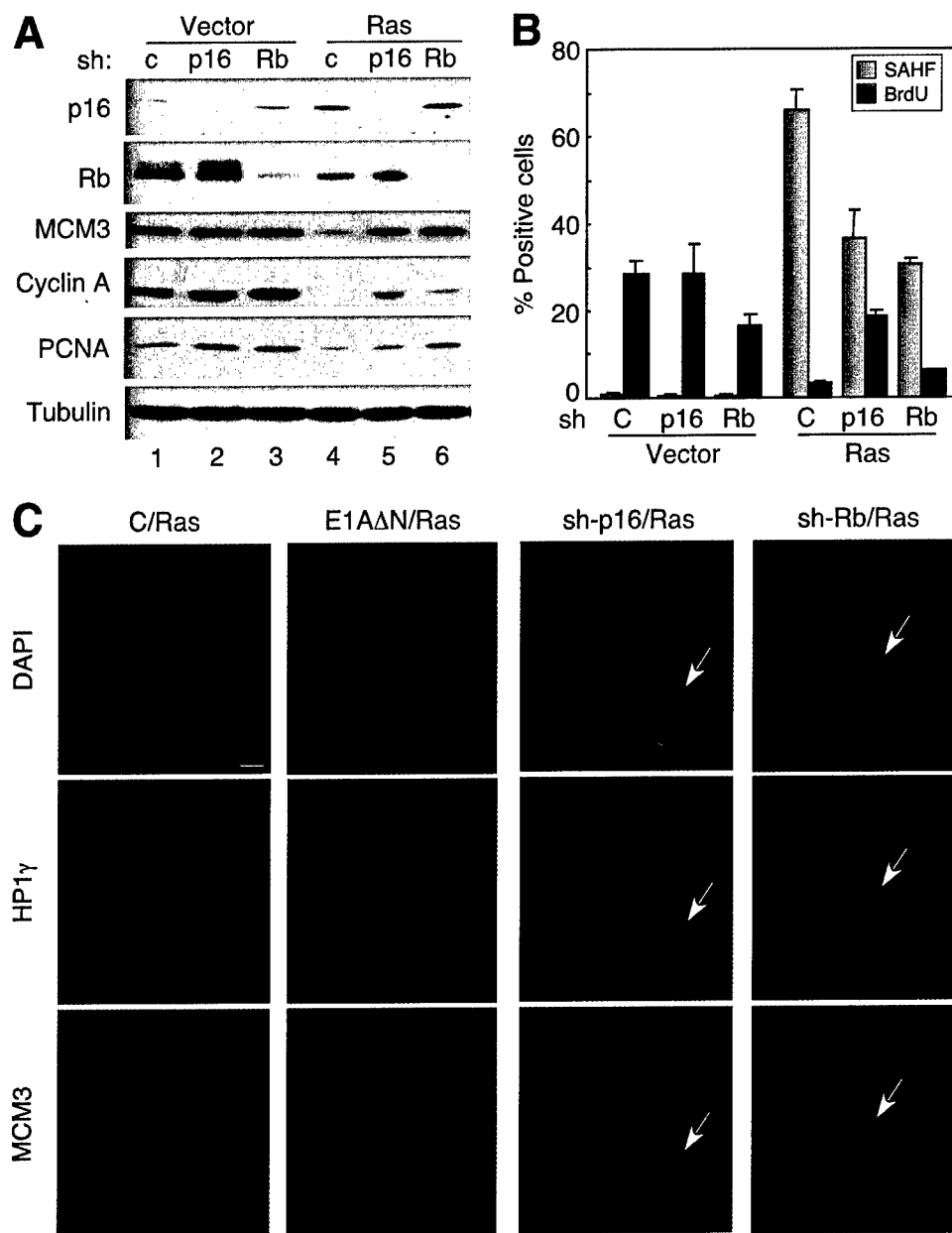


Figure 7. The p16<sup>INK4a</sup>/Rb Pathway Is Crucial for SAHF Formation and Gene Silencing

(A) The expression of the indicated protein was determined by immunoblotting of extracts from IMR90 cells expressing empty vector or H-rasV12 (Ras) in combination with short hairpin RNAs (sh) against p16<sup>INK4a</sup> or Rb, as well as control vector (c) at day 5 (see Figure 1B).

(B) The cells were also assessed for SAHF formation and BrdU incorporation at the same time point as in (A).

(C) Indirect immunofluorescent images for HP1γ and MCM3 of IMR90 cells expressing the indicated genes. Cells were at PS day 8 (PS8) (see Figure 1B). The nuclei, which failed to form SAHFs in sh-p16/Ras and sh-Rb/Ras, are indicated with arrows. DNA was counterstained by DAPI. Scale bar is equal to 10 μm.

versible cell cycle arrest and imply that, in the absence of SAHF formation, this arrest is more difficult to maintain.

#### Quiescence Versus Senescence

Much of what we know concerning the regulation of E2F activity comes from studies examining cell cycle transitions into and out of a quiescent state (Trimarchi and Lees, 2002). These transitions are controlled in a reversible manner, in part, by the competing action of HATs and HDACs on the histones of E2F target promot-

ers (see Introduction). In this study, we compared the physical state and regulation of E2F target genes in quiescent and senescent cells. In both cell states, the amount of K9-acetylated histone H3 that associates with E2F target promoters declines, consistent with the downregulation of transcription that accompanies cell cycle exit. However, in senescent IMR90 cells, histone H3 acetylation is ultimately replaced by methylation at lysine 9, an apparently irreversible modification that prevents acetylation by HATs and is barely observed on

E2F-responsive promoters in quiescent cells (see Figure 4C). Methylated lysine 9 forms a docking site for HP1 proteins and, accordingly, HP1 $\gamma$  preferentially associates with E2F target promoters in senescent cells. These modifications are predicted to form a "lock" on the transcription of E2F responsive promoters, making them less accessible to the transcription machinery (Dillon and Festenstein, 2002; Trimarchi and Lees, 2002). Accordingly, several E2F-responsive genes in senescent cells are stably repressed and insensitive to enforced E2F expression relative to quiescent cells (see Figure 5). Although it remains to be determined whether every E2F target gene behaves as those studied here, their transition to a heterochromatin-like organization may contribute to the insensitivity of senescent cells to mitogenic signals and the apparent irreversibility of the senescence process.

What determines whether a cell enters quiescence or senescence? Although many factors undoubtedly play a role, our results imply that Rb is crucial for this decision. Interestingly, although the current paradigm for Rb action suggests that Rb negatively regulates normal cell cycle transitions by recruiting HDACs to transcriptionally repress E2F target genes, recent reports have been unable to detect Rb on E2F target promoters in G1 and quiescent cells (Rayman et al., 2002; Takahashi et al., 2000). In contrast, p107 and p130 were readily detected. While we confirm these reports, we also show that Rb appears on some E2F target promoters in senescent cells. Since Rb can associate with certain histone methyltransferases and the HP1 proteins (Nielsen et al., 2001b), the simplest model to explain our results is that Rb acts directly on E2F target promoters to nucleate regions of heterochromatin leading to the silencing of E2F target genes and perhaps spreading to other euchromatic loci. However, we cannot exclude the possibility that Rb acts at other sites in the genome or controls the process indirectly, perhaps by influencing the expression or activity of chromatin remodeling factors.

Our model implies that the decision to enter senescence is determined, in part, by a histone methyltransferase (HMT) that acts with Rb and HP1 proteins to alter chromatin structure and silence E2F targets genes. One candidate for this molecule is SUV39H1, which is a known regulator of PEV in *Drosophila* and required for the maintenance of pericentric heterochromatin in mammalian cells (Peters et al., 2001). Consistent with this possibility, SUV39H1 can interact with Rb and affect the expression of some E2F target genes (Nielsen et al., 2001b). Moreover, enforced expression of SUV39H1 induces a senescent-like arrest in IMR90 fibroblasts in a manner that depends on its methyltransferase activity (M.N. and S.W.L., unpublished data). However, Rb can interact with other histone methyltransferases (Steele-Perkins et al., 2001), and Suv39h is not required for all types of heterochromatin formation (Peters et al., 2002). Furthermore, *Suv39h1/h2*-deficient MEFs arrest in response to oncogenic *ras* (M.N., T. Jenuwein, and S.W.L., unpublished data). Therefore, another HMT may act alone or together with SUV39H1 to mediate SAHF formation and E2F gene silencing during senescence. Presumably these interactions do not occur as cells exit the cell cycle into a quiescent state.

### Senescence, Rb, and Tumor Suppression

A large body of work implicates the p53 and Rb pathways in cellular senescence and, as such, the process may function as a natural brake to tumor development. In this study, we show that Rb contributes to senescence by promoting SAHF formation and silencing E2F target genes. Interestingly, in IMR90 cells, Rb is not essential for all aspects of the senescence program, including some of the morphological changes, the accumulation of SA- $\beta$ -gal activity, and the ultimate exit from the cell cycle. We have proposed that senescence in human diploid fibroblasts involves the combined activities of the p16/Rb pathway, p53, and the promyelocytic leukemia protein (PML) (Ferbeyre et al., 2000), and perhaps p53, PML, or some other activity controls aspects of the senescent morphology and the initial cell cycle exit. However, loss of *INK4a* alone appears sufficient to override senescence in some human fibroblast strains (Brookes et al., 2002), and IMR90 cells with defects in the Rb pathway show a delay in cell cycle exit following expression of oncogenic *ras* and entirely fail to senesce in response to p16<sup>INK4a</sup>.

Presumably, the failure to silence E2F target genes reduces the probability that a damaged cell undergoes senescence or, alternatively, makes the arrest more difficult to sustain. Consistent with this view, a critical difference between IMR90 and WI38 fibroblasts (which form SAHFs) and BJ fibroblasts and MEFs (which apparently do not) appears to be the relative importance of the p16-Rb pathway in controlling the arrest. Hence, IMR90 and WI38 cells have a robust p16<sup>INK4a</sup> response during senescence (e.g., Serrano et al., 1997), and studies using RNA interference indicate that p16 is responsible for their inability to cycle upon p53 inactivation (Beauséjour et al., submitted). In contrast, p16<sup>INK4a</sup> is poorly induced in senescent BJ fibroblasts and appears dispensable for senescence in MEFs (Beauséjour et al., submitted; Lowe and Sherr, 2003). Our results provide a potential mechanism for these observations, implying that p16<sup>INK4a</sup> upregulation during senescence engages the Rb pathway to produce a permanent arrest by altering the chromatin state of growth regulatory genes. The failure of these processes in cells sustaining *INK4a* or *Rb* mutations may lead to cancer progression or increase the likelihood of tumor relapse from a dormant state.

Mutations arising in malignant tumors often pinpoint processes that must be altered during tumor evolution. Although cellular senescence provides an important brake to human cell transformation in cell culture (Hahn and Weinberg, 2002), its contribution to tumor suppression in vivo is poorly defined. Nevertheless, the role of the p16-Rb pathway in cellular senescence coupled with its frequent mutation in human cancers provides strong circumstantial evidence that the senescence program limits the development of malignant tumors. Moreover, many anticancer agents can induce cellular senescence, and the p16<sup>INK4a</sup>/Rb pathway is important for drug-induced senescence in vivo (Chang et al., 1999; Schmitt et al., 2002; te Poele et al., 2002). Our results provide some of the first mechanistic insights into the effector mechanisms of senescence and, as such, may identify processes that control cancer progression and whether tumors undergo a sustained response to therapy.

## Experimental Procedures

### Retroviral Vectors

The following retroviral vectors were used in this study: pBabe-Puro (Oncogenic *ras* (H-RasV12) and *MEK1* Q56P) (Lin et al., 1998); pWZL Hygro (H-RasV12, p16<sup>INK4a</sup>, and human p53<sup>175H</sup>) (Serrano et al., 1997); pLPC-Puro (EGFP-tagged human *HP1* $\beta$  cDNA and *E1A* and *E1A*  $\Delta$ M) (Samuelson and Lowe, 1997); and pMSCV-puro (Clontech) (*INK4a* and *Rb* shRNAs). Additional information can be found in the Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>.

### Cell Culture and Gene Transfer

Human diploid IMR90 fibroblasts (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics. Retroviruses were packed using the Phoenix cells (G. Nolan, Stanford University, CA) and infections were performed as described (Serrano et al., 1997) except that amphotropic viruses were used to produce cells for the ChIP assays and RNAi experiments. The infected population was selected using either 2  $\mu$ g/ml puromycin (Sigma) for 2–3 days or 100  $\mu$ g/ml hygromycin B (Roche) for 2–3 days. For coinfection of puromycin- and hygromycin-selectable vectors, cells were selected with puromycin first for 2 days, followed by hygromycin selection for another 2 days. Adenoviruses were introduced into quiescent and senescent IMR90 cells at a multiplicity of infection (MOI) of 15 plaque-forming units (PFU) cell<sup>-1</sup>. For the experiments in Figure 5B, Ad-GFP was used at an MOI of 4 PFU cell<sup>-1</sup> and Ad-E2F1 was used in a range of 0.03 and 15 PFU cell<sup>-1</sup>.

### Cell Proliferation and SA- $\beta$ -gal Assays

IMR90 cells were plated on coverslips and subsequently labeled with 5-Bromo-2'-deoxyuridine (BrdU, 100  $\mu$ g/ml, Sigma) and 5-fluor-2'-deoxyuridine (FdU, 10 mg/ml, Sigma) for 6 hr. Nuclei incorporating BrdU were visualized by immunolabeling using anti-BrdU antibody (Pharmingen, 1:400) as previously described (Humbert et al., 2000). SA- $\beta$ -gal activity was detected as previously described (Serrano et al., 1997). DNA was visualized by DAPI (1  $\mu$ g/ml) after permeabilization with 0.2% Triton X-100/PBS.

### Immunolabeling, Electron Microscopy, and RNA FISH

Immunofluorescence studies were performed using standard procedures (see Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>) using the following primary antibodies: anti-HP1 $\alpha$  antibody (1:200, provided by W.C. Earnshaw); anti-HP1 $\beta$  antibody (1MOD-1A9, 1:500, provided by P. Chambon); anti-HP1 $\gamma$  antibody (2MOD-1G6, 1:5000, provided by P. Chambon); anti-K9, 14Ac-H3 antibody (Upstate, 1:1000); anti-K9M-H3 antibody (provided by C.D. Allis and Upstate, 1:1500); anti-K4M-H3 antibody (Upstate, 1:700); anti-MCM3 antibody (provided by B. Stillman, 1:200); and anti-Rb antibody (G3-245, Pharmingen, 1:100) together with XZ-55 and C36 hybridoma supernatant (1:50).

Electron microscopy was performed as previously described (Woo et al., 1998). For DNA/RNA labeling, IMR90 cells, cultured on Thermanox (Electron Microscopy Sciences) coverslips, were fixed with 1.5% glutaraldehyde in PBS, ethanol dehydrated at 0 to minus 20°C, embedded in Lowicryl K4M resin (Electron Microscopy Sciences) and polymerized at -36°C. Thin sections cut en face at 100 nm thickness were collected on uncoated nickel grids, incubated in essentially globulin-free 1% BSA (Sigma) in PBS, incubated in drops of primary mouse monoclonal antibody to DNA (AC-30-10 Maine Biotechnology Services) diluted in PBS, for one hour at 22°C and then rinsed in PBS and incubated for 30 min in drops of secondary antibodies conjugated to 10 nm colloidal gold (Pharmacia). After immunogold labeling, sections were rinsed in distilled water, air dried and briefly counterstained with 3% aqueous uranyl acetate. RNase-gold solution was freshly prepared by conjugating RNase T<sub>1</sub> from *Aspergillus oryzae* (Sigma) to 20 nm colloidal gold particles (Chenicle and Bendayan, 1990). Sections were incubated in RNase gold for 15 min at 37°C, rinsed, and stained as above.

RNA FISH was performed as described elsewhere (Clemson et al., 1996) (see Supplemental Data available at <http://www.cell.com/cgi/content/full/113/6/703/DC1>) using bacterial artificial chromo-

some (BAC) clones CTD-2097K16 and CTD-2217D23 (ResGen) as probes for *INK4a* and *cyclin A* genes, respectively.

### Micrococcal Nuclease Assay

Cells were permeabilized with 0.01% L- $\alpha$ -lysophosphatidylcholine (Sigma) in 150 mM sucrose, 80 mM KCl, 35 mM HEPES [pH 7.4], 5 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM MgCl<sub>2</sub>, and 0.5 mM CaCl<sub>2</sub> for 90 s, followed by digestion with 2 U/ml micrococcal nuclease (Sigma) in 20 mM sucrose, 50 mM Tris [pH 7.5], 50 mM NaCl, and 2 mM CaCl<sub>2</sub> at room temperature for various times. DNA was isolated and subjected to 0.8% agarose gel electrophoresis.

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations were performed as described previously (Nahle et al., 2002) using anti-K9/14Ac-H3 (Upstate), K9M-H3 (ab7312, Abcam, Upstate, or provided by C.D. Allis), HP1 $\gamma$  (Chemicon), Rb (C-15, Santa Cruz), p107 (C-18, Santa Cruz), and p130 (C-20, Santa Cruz) antibodies. DNA released from precipitated complexes was amplified using sequence specific primers by PCR. PCR products were labeled by [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia), separated on a 4% nondenaturing polyacrylamide gel. The primer sets used were: promoter regions of *cyclin A*, *PCNA*, *p16INK4a*, *stromelysin-1*, and  $\beta$ -actin (sequences are available from the authors upon request).

### Western Blotting

Western blotting analysis was carried out on 20  $\mu$ g whole-cell lysate by using enhanced chemiluminescence (ECL; Amersham) detection as previously described (Serrano et al., 1997). Blots were probed with the following antibodies: anti-p16 antibody (NA29, Oncogene, 1:200); anti-Ras antibody (OP23, Oncogene, 1:300); anti-Rb antibody (G3-245, Pharmingen, 1:1000) together with XZ-55 hybridoma supernatant (1:100); anti-E2F1 antibody (KH95, Santa Cruz, 1:200); anti-cyclin A antibody (Sigma, 1:1000); anti-PCNA antibody (PC10 hybridoma supernatant, 1:1000); anti-MCM3 antibody (provided by B. Stillman, 1:500); anti-E1A antibody (M73 hybridoma supernatant, 1:300); anti-p53 (DO1, Oncogene, 1:1000); and anti- $\alpha$ -tubulin (B-5-1-2, Sigma, 1:2000).

### Real-Time PCR

Total RNA was isolated by RNeasy Mini Kit (Qiagen) and was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Gene-specific TaqMan primer sets were designed using Primer Express 1.5 (sequences are available from the authors upon request). Real-time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and  $\beta$ -actin serves as an endogenous normalization control. Sequence Detector software (version 1.7) was utilized for data analysis and relative fold induction was determined by the comparative threshold cycle method.

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